# ESTIMATION AND COMPARISON OF UNSTIMULATED SALIVARY FLOW RATE, PH, STREPTOCOCCUS SALIVARIUS LEVEL ALONG WITH BUCCAL EPITHELIAL MICRONUCLEI CHANGES AMONG THE POPULATION RESIDING AROUND THE CELL PHONE TOWERS WITH VARYING POWER DENSITY – A COMPARATIVE STUDY

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## 1. Introduction:

Electromagnetic field (EMF) radiation is ubiquitous [1]. The radiofrequency (RF) waves used in wireless communication are non-ionizing as they do not ionize biological matter [2]. In recent times, the use of mobile phones has increased tremendously. Consequently, there has been an apparent increase in the number of cell phone towers by various network providers. This as well as the continuous whole-body nature of continuous exposure may constitute a public health risk to humans [3], which still require a scientific assessment.

When a radiofrequency current is supplied to an antenna, the radiofrequency EMF propagates through space with a frequency of 100 kHz to 300 GHz. In particular, precise information regarding the mechanism of biological effects by RF-EMF radiation has not yet been completely elucidated. The continuous exposure to EMF from the cell phone towers may have acute thermal and chronic non-thermal adverse effects similar to EMF from cell phones. Every interaction between radiofrequency fields and living tissues causes an energy transfer resulting in a temperature rise [4]. Increased body temperature is stabilized and alleviated by blood circulation. Although non-thermal effects do not raise the body temperature sufficiently to impair the structure of tissues, their effects can still be seen as an increase in free radical production in tissues [5].

Most animal studies have found no evidence of *in vitro* RF-induced genetic damage at non-thermal exposure regimes [5,6]. Although animal studies provide qualitative information about possible effects, these findings cannot be extended to generate a credible estimate of human risk.

Literature search revealed people residing around cell phone towers show statistically meaningful salivary changes. Augner et al. (2010) observed an increase in salivary cortisol, alpha-amylase and IgA in high exposure conditions from cell phone towers [7]. Singh K et al. (2016) observed a decrease in stimulated salivary flow volume among the people residing near base transceiver station [8]. Thamilselvan S et al. conducted a cross-sectional study to detect the presence of micronuclei in the buccal epithelial cells among a population residing within 10 - 25 m around three cell phone towers at Chennai, India. The results of the study shows that 55% of the population had the presence of micronuclei in at least one field. In the age group under 10 years, 47·1% shows the presence of micronuclei [9]. However, the subject

included in the study was based on distance and the means of measuring the distance was not clearly defined. Further, the cell phone tower characteristics with respect to the variation in the number and the type of antenna was also not considered before saliva collection and only four cell phone towers were included in the study.

Since, there are two different schools of thought concerning the safety of the EMF radiation from the cell phone towers, it is prudent to arrive at a standard method for conducting research in human population residing near and around the cell phone towers especially involving human biological sample. The researchers prefer arbitrary distance-based classification to study changes in human biological sample among the population residing around the cell phone towers, despite variations in cell phone tower characteristics with respect to number of antennas, type of antennas, Electric Field Strength (EF), Magnetic Field strength (MF) and Power Density (PD). Power density is the rate of flow of electromagnetic energy or power per unit area. It is expressed as $\mu W/cm^2$ [10].

Literature search revealed no data with respect to the changes in unstimulated salivary flow rate, pH, *Streptococcus salivarius* and micronuclei changes in buccal epithelial cells among population residing around the cell phone towers with low, medium and high-power density.

## 2. Aim and objectives:

### Aim:

To estimate and compare the unstimulated salivary flow rate, pH, *Streptococcus salivarius* and buccal epithelial micronuclei count among the population residing around the cell phone towers with varying power density.

### Objectives:

1. To estimate and compare the electric field strength, magnetic field strength and power density around the cell phone towers at 50 m and 100 m in four directions.
2. To develop a power density-based classification using the data collected around the cell phone towers at 50 m and 100 m in four directions.
3. To estimate and compare the unstimulated salivary flow rate, pH, *Streptococcus salivarius* level and buccal epithelial micronuclei count among the population residing around the cell phone towers with low, medium and high-power density.

## 3. Research question:

Is there any changes in the unstimulated salivary flow rate, pH, *Streptococcus salivarius* and buccal epithelial micronuclei count among the population residing around the cell phone towers with low, medium and high power density?

## 4. Review of literature:

### 4.1 International studies

Augner C et al. (2010) studied the effects of EMF radiation from cell phone tower in 57 participants in three different experimental scenarios on the salivary alpha amylase, cortisol and IgA on 50-minute exposure session with low (5.2 $\mu W/m^2$), medium (153.6 $\mu W/m^2$) and high PD (2126.8 $\mu W/m^2$) exposure. He observed statistically significant increase in cortisol and alpha amylase due to high EMF radiation whereas IgA is not statistically significant [7].

Claudio Gómez-Perretta et al. (2013) studied the subjective symptoms related to GSM radiation from the cell phone towers in 88 participants and observed that they were more prone to symptoms such as loss of appetite, vertigo, sleep problems, irritability

and lack of appetite. The results of this study partially confirmed the microwave sickness resulting from exposure to EMF radiation from cell phone towers [11].

### 4.2 Indian studies

Singh K et al. (2016) conducted a study at Jaipur, India, to determine the effects of EMF radiation on stimulated salivary flow volume, pH and other health related problems like sleep disturbance, headache and irritability among 40 participants residing near 4 cell phone towers and compared with another group located 1 km away from the cell phone tower. The results of the showed reduction in salivary flow volume and no statistically significant difference in pH [8].

Thamilselvan S et al. (2021) conducted an observational study to detect the presence of micronuclei in the buccal epithelial cells among the population residing within 10–25 m around three cell phone towers at Chennai, India. The results of the study show that 55% of the population had the presence of micronuclei in at least one field. In the age group under 10 years, 47.1% showed the presence of micronuclei [9].

## 5. Materials and methods:

### 5.1 ESTIMATION AND COMPARISON OF THE ELECTRIC FIELD STRENGTH, MAGNETIC FIELD STRENGTH AND POWER DENSITY AROUND THE CELL PHONE TOWERS AT 50 M AND 100 M IN FOUR DIRECTIONS:

**5.1.1 Study characteristics:** After obtaining ethical clearance of Sri Balaji Vidyapeeth (IEC No. PhD/2016/03/02), the observational study was conducted in Pondicherry, India. The study was conducted in an urban setup around residential houses with a maximum of two to three floors with background radiation. The cell phone towers included in the study were roof mounted and ground-based containing disk and sector antennas at different positions set by the service provider.

**5.1.2 Sample size calculation:** A total of 16 cell phone towers in Puducherry, India, were selected using convenience sampling. The sample size was calculated by the following formula.

$$Sample\ size\ (n) = (1 + \sqrt{g-1}\,)\frac{(Z_{1-\alpha/2}+(Z_{1-\beta})^2}{d^3} + \frac{Z^2{}_{1-\alpha/2}\sqrt{g-1}}{2(1+\sqrt{g-1})}$$

where α was the Type 1 error rate, $\beta$ was the Type 2 error rate, $d$ was the expected effect size, $g$ was the number of groups to compare and $Z$ was the value from the standard normal distribution reflecting the confidence interval, $Z$ = 1.96 for 95% confidence.

**5.1.3 Geographical mapping of cell phone towers:** Based on their GPS coordinates, the geographical mapping of the selected 16 cell phone towers was performed in order to calculate the distance between each tower (Figure 1). For geographical mapping of cell phone towers, a Garmin etrex 20x device (Garmin, New Taipei City, Taiwan, China) was used. The device provides the global positioning system (GPS), global navigation satellite system (GLONASS) and wide area augmentation system (WAAS) and a greater accuracy of ±3 m (Figure 2a). Using the two sets of cell phone tower coordinates in the Online Federal Communication Commission tool (FCC) [12], the distance between cell phone towers was calculated and found to be more than 200 m (Figure 2b).

**5.1.4 Study groups:** Based on the number of antennas, the cell phone towers were categorized into four groups, where group I consisted of towers with 1–5 antennas, group II had towers with 6–10 antennas, towers with 11–15 antennas are considered in group III, and lastly, tower clusters with more than 15 antennas were in group IV. Cell phone towers with both disk and sector antennas were included in the study.

**5.1.5 Measurement of Electric Field Strength, Magnetic Field Strength and Power Density around the cell phone towers:**

The distances, 50 m and 100 m, were measured using the Laser range finder (Bosch Professional GLM 250 VF, Switzerland) directed towards the lowest antenna in the cell phone tower. The measuring range of this tripod-mounted device was 0.05–250 m with a high accuracy of ± 1 mm (Figure 2c). The EF, MF and PD were

measured at 50 and 100 m on all four sides around the cell phone tower using the pre-calibrated HTC EMF 532 RF three-axis field strength meter (HTC, Mumbai, India) (Figure 2d). This CE-certified HTC EMF 523 RF meter was designed for measuring and monitoring radiofrequency electromagnetic field strength, precisely calibrated in the frequency range of 50 MHz–3.5 GHZ. It used an isotropic triaxial measurement with a sample rate of 3 times per second. The dynamic range of the meter was typically 75 dB. The absolute error at 1 V/m and 2.45 GHz was ± 1.0 dB. The device measured the electric filed strength directly and displayed the calculated magnetic field strength and power density. The device was held at one arm's length at chest height for a minimum of at least two minutes and the maximum average values of EF, MF and PD were measured. Three consecutive measurements were taken and the third value was recorded. Three measurements were taken to ensure that the EF, MF and PD values were genuinely representative of that particular area. All the measurements were taken during the daytime between 9 a.m. and 11 a.m. No other EMF producing devices or sources such as mobile phones and electrical lines were present while recording the data. The EMF measurement on four sides around the cell phone tower followed the same pattern of measurement, namely, north, east, south and west. The max average values of the EF, MF and PD were recorded at 50 and 100 m on all four sides.

**5.1.6 Statistical Analysis:** The data were subjected to statistical analysis. One-way ANOVA was performed to compare the EF, MF, and the PD among the four study groups and different sides using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA). The Unpaired *t* test was used for Intragroup comparison.

Inter- and intragroup comparative analysis of electric field strength, magnetic field strength and power density among the study group at different distances is presented in Table 1.

**Table 1. Inter- and intragroup comparative analysis of electric field strength, magnetic field strength and power density among the study group at different distances.**

| SG | EF | | *p* value ‡ | MF | | *p* value ‡ | PD | | *p* value ‡ |
|---|---|---|---|---|---|---|---|---|---|
| | 50 m | 100 m | | 50 m | 100 m | | 50 m | 100 m | |
| I | 588.88 ± 426.87 | 525.79 ± 458.00 | 0.690 | 1590.90 ± 1116.30 | 1345.17 ± 1166.39 | 0.547 | 0.07 ± 0.09 | 0.10 ± 0.11 | 0.519 |
| II | 962.65 ± 1528.52 | 657.68 ± 515.25 | 0.455 | 1545.02 ± 1083.03 | 1784.84 ± 1406.43 | 0.593 | 0.09 ± 0.08 | 0.15 ± 0.21 | 0.283 |
| III | 555.19 ± 292.34 | 661.48 ± 358.62 | 0.365 | 1440.84 ± 832.57 | 2085.10 ± 1157.29 | 0.081 | 0.06 ± 0.06 | 0.10 ± 0.08 | 0.168 |
| IV | 1448.51 ± 2566.34 | 574.34 ± 316.66 | 0.186 | 2221.28 ± 884.85 | 1616.91 ± 745.03 | 0.045 * | 0.12 ± 0.09 | 0.07 ± 0.06 | 0.049 * |
| *p* value † | 0.315 | 0.754 | | 0.116 | 0.327 | | 0.190 | 0.362 | |

Note: † One-Way ANOVA; ‡ Unpaired *t* Test; * $p < 0.05$; SG—Study Groups; EF—Electric field strength measured in mV/m; MF—Magnetic field strength measured in μA/m; PD—Power density measured in $\mu W/cm^2$; Results are expressed in mean ± standard deviation.

The inter- and intragroup comparative analysis of EF, MF and PD among the study groups at different directions is presented in Table 2. Group-wise comparisons of EF (Figure 3), MF (Figure 4) and PD (Figure 5) around the cell phone towers at 50 m and 100 m on all four sides are shown in the radar chart. With respect to the EF, MF, and the PD in all four directions around the cell phone tower at 50 and 100 m, a typical bow and tie pattern is observed among all four Groups. On three sides, it is less and on the fourth side, the EF, MF, and PD are quite high, hence the bow and tie pattern. Hence, the EF, MF and PD in all four directions are not consistent with the distance in the present study. Due to this variation in all four sides around the cell phone towers, the distance-based grouping cannot be used in research involving biological samples for this population. Hence, there is a need for a standard alternative reliable method of classification.

The inter- and intragroup comparative analysis of EF, MF and PD among the study groups at different directions is presented in Table 2.

**Table 2. Inter- and intragroup comparative analysis of Electric field strength, magnetic field strength and power density among the study groups at different directions.**

| SG | Electric Field Strength (EF) | | | | *p* Value | Magnetic Field Strength (MF) | | | | *p* Value | Power Density (PD) | | | | *p* Value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | North | South | East | West | | North | South | East | West | | North | South | East | West | |
| I | 676.95 ± 470.22 | 581.06 ± 359.06 | 424.52 ± 426.37 | 546.82 ± 524.80 | 0.732 | 1722.53 ± 1190.4 | 1577.47 ± 925.56 | 1108.45 ± 1083.08 | 1463.69 ± 1404.32 | 0.750 | 0.106 ± 0.11 | 0.086 ± 0.09 | 0.077 ± 0.11 | 0.090 ± 0.11 | 0.958 |
| II | 1551.08 ± 2048.39 | 463.96 ± 299.98 | 502.26 ± 476.36 | 723.36 ± 536.29 | 0.187 | 2239.77 ± 955.32 | 1331.31 ± 978.84 | 1356.85 ± 1277.38 | 1731.79 ± 1642.25 | 0.439 | 0.220 ± 0.22 | 0.066 ± 0.07 | 0.076 ± 0.10 | 0.132 ± 0.18 | 0.215 |
| III | 609.61 ± 170.58 | 681.52 ± 510.99 | 428.91 ± 230.82 | 713.30 ± 269.52 | 0.312 | 1622.88 ± 471.32 | 1898.16 ± 1333.27 | 1626.15 ± 1496.50 | 1904.70 ± 709.08 | 0.914 | 0.079 ± 0.049 | 0.115 ± 0.130 | 0.043 ± 0.035 | 0.103 ± 0.069 | 0.311 |
| IV | 651.06 ± 199.95 | 1816.22 ± 3718.41 | 893.03 ± 355.12 | 685.39 ± 427.14 | 0.574 | 1736.51 ± 522.08 | 1555.037 ± 924.40 | 2386.18 ± 917.10 | 1998.66 ± 932.94 | 0.245 | 0.076 ± 0.042 | 0.070 ± 0.075 | 0.143 ± 0.092 | 0.112 ± 0.102 | 0.260 |
| *p* Value | 0.245 | 0.464 | 0.064 | 0.852 | | 0.467 | 0.760 | 0.197 | 0.831 | | 0.118 | 0.734 | 0.185 | 0.920 | |

Note: SG—Study Groups; EF—Electric field strength measured in mV/m; MF—Magnetic field strength measured in μA/m; PD—Power density measured in μW/cm$^2$; Results are expressed in mean ± standard deviation.

**5.2 DEVELOPMENT OF POWER DENSITY BASED CLASSIFICATIONS:** Since the entire dataset with respect to power density from all four groups around cell phone towers is available in the present study, we have developed a new power density-based classification as low, medium and high, based on quantile after excluding the outliers (Table 3). The power density data from the present study are arranged in ascending order from lowest to the highest and divided into three equal parts based on quantiles. To minimize the bias in forming the groups, we opted to use a quantile, as this statistical concept possesses an objective definition and a clear meaning. For categorizing into low, medium and high, the concept of quantiles is ideal to generate ordinal data and it assigns the same number of data values to each class. There are no empty classes or classes with too few or too many values. The drawback of the quantile classification is that features with widely different values can end in the same

group, while similar values can be placed adjacent groups. This distortion is minimized by classifying PD into three groups, labelled as low, medium and high, instead of into two groups.

**Table 3. Developed classification based on the power density**

| Groups | Power Density (μW/cm$^2$) |
|---|---|
| Low | 0.001–0.029 |
| Medium | 0.03–0.099 |
| High | 0.1–0.355 |

## 5.3 ESTIMATION AND COMPARISON OF UNSTIMULATED SALIVARY FLOW RATE, PH, *STREPTOCOCCUS SALIVARIUS* LEVEL ALONG WITH BUCCAL EPITHELIAL MICRONUCLEI CHANGES AMONG THE POPULATION RESIDING AROUND THE CELL PHONE TOWERS WITH LOW, MEDIUM AND HIGH-POWER DENSITY:

### 5.3.1 Study characteristics:

The comparative study was conducted after obtaining the Institutional Ethical Clearance from Sri Balaji Vidyapeeth (IEC No. PhD/2016/03/02). The study was conducted in and around Puducherry and Tamil Nadu in an urban setup in residential houses with a maximum of 2 to 3 floors. The cell phone towers included in the study were roof-mounted and ground-based, containing disk and sector antennas at different positions within the cell phone tower set by the service provider.

### 5.3.2 Sample size:

A total of 60 participants were included in the study with 20 participants in three groups. The sample size was calculated using G power software with 95% power, effect size (d) 1.1121, $\alpha = 0.05$. The study participants were recruited using convenience sampling.

### 5.3.3 Inclusion criteria:

Male and female adults aged between 18 – 30 years, residing in their house for a period of at least 8 hr per day and over 2 years around the cell phone towers were included in the study.

In the low power density group, male and female study participants residing in low power density around the cell phone towers were included. **Low power density corresponds to 0.001 – 0.029 μW/cm$^2$**.

In the medium power density group, male and female study participants residing in medium power density around the cell phone towers were included. **Medium power density corresponds to 0.03 – 0.099 µW/cm$^2$**.

In the high-power density group, male and female study participants residing in high power density around the cell phone towers were included. **High power density corresponds to 0.1 – 0.355 µW/cm$^2$**.

### 5.3.4 Exclusion criteria

The study participants with systemic diseases that alter the salivary flow rate such as Diabetes mellitus, hypertension, sialorrhoea, etc., were excluded from the study. The participants on medications which alter the salivary flow rate such as antihistamines were excluded from the study. The participants on long-term medication, history of smoking, alcoholism, chemotherapy and radiotherapy of the head and neck region were also excluded from the study. The study participants with dental caries, restored tooth, fractured tooth, a periodontal pocket of more than 3 mm, oral mucosal lesions, dental appliances and dentures were excluded from the study.

### 5.3.5 POWER DENSITY MEASUREMENT INSIDE THE HOUSE:

The power density was measured inside the house using the HTC EMF device and the participants were categorized into low, medium and high. The Radiofrequency EMF-producing devices such as Bluetooth, wi-fi, personal hot spot, mobile phones and WLAN were turned off before recording the power density inside the house.

### 5.3.6 EXTRAORAL AND INTRAORAL ASSESSMENT OF STUDY PARTICIPANTS:

After a thorough history, the extraoral assessment was done using WHO Oral Health Assessment Form 1997 [13]. The intraoral assessment was done using WHO Oral Health Assessment Form for adults 2013 [14]. CPITN C probe was used for assessing the periodontal status of the study participants. One observer was involved in the extraoral and intraoral assessment. Cronbach's alpha 1.00 suggested excellent intra-observer reliability as shown in table 4.

**Table 4. Intra-observer reliability**

| Reliability Statistics | |
|---|---|
| N of items | Cronbach's alpha |
| 233 | 1.00 |

### 5.3.7 A QUESTIONNAIRE TO DETERMINE THE DIFFERENT EMF-PRODUCING DEVICES USED INSIDE THE HOUSE AND THEIR WEEKLY DURATION OF USE

A rationale 44-point self-constructed and validated questionnaire was used for assessing the different EMF-producing devices inside the house. The questionnaire was subjected to peer validation by 25 faculties and 2 experts for content validity before administering it to the study participants. The questionnaire also collected information regarding the frequency of use in terms of the number of times in a day and week along with the hours of use. The questionnaire also has an additional table where the study participants can add other unlisted EMF-producing devices used inside the house, which was subjected to the approval of the principal investigator.

This study is unique as it considered all the EMF producing devices inside the house. This was done to study the total mean weekly use of different EMF devices in terms of hours so that a comparison can be made among the three groups.

### 5.3.8 MEASUREMENT OF UNSTIMULATED SALIVARY FLOW RATE:

The study participants were instructed not to eat or drink one hour before the saliva collection. Strict aseptic measures were followed during saliva collection. All the unstimulated salivary samples were collected inside the house between 9 am – 11 am to prevent diurnal variation. The participants were instructed to rinse their mouths with distilled water before saliva collection. The unstimulated whole saliva was collected using the spitting method. The saliva was allowed to collect on the floor of the mouth and spit into the pre-weighed container at the end of one minute. The total duration of saliva collection was 5 mins. The post-weight of the container with saliva was recorded and the unstimulated salivary flow rate per minute was calculated using the gravimetric method (1 g of saliva is equal to 1 ml) [15]. The pre-weight and post-weight of the container were measured using a precision balance (Wensar PGB 220, India) with a readability of 0.001 g (1 mg), repeatability of ± 0.001 g, automatic

external calibration with quick weighing and accurate results (Figure 6). In all participants, both pre-weight and post-weight of the container were measured with the lid. The collected saliva for salivary flow rate estimation was immediately stored in the ice pack, transported and stored at -20 degrees and later at -80 degrees for assessing *Streptococcus salivarius* level using Real-Time PCR.

### 5.3.9 MICRONUCLEI COUNT FROM BUCCAL EPITHELIAL SMEAR

The study participants were instructed to rinse their mouths with water. The buccal epithelial cytosmear was obtained from the left buccal mucosa using the cytobrush (Pap Procer, Bangalore, India) (Figure 7) in a slow, gentle, rotatory motion from up to down direction without contacting the tooth surface by the principal investigator. The cells were transferred to a clean slide and immediately fixed with Propan-2-ol and ether (Sytofix, Bangalore, India). The fixed buccal smear from the study participants house was transported to the lab for Papanicolaou (PAP) staining.

**PAP staining method**

The buccal epithelial cells of the study participants were stained with PAP stain to visualize the micronuclei. The slide was dipped in the following solutions in sequential order (Figure 8a).

1. 70% Ethanol for 1 min
2. 50 % Ethanol for 1 min
3. Distilled water – 5 dips
4. Harris Hematoxylin for 4 min
5. Distilled water – 5 dips
6. 0.5% acid alcohol – 1 dip
7. Water for 1 min
8. Lithium carbonate for 1.5 min
9. Water – Few dips
10. 70% Ethanol for 2 min
11. 90% Ethanol for 2 min
12. Orange G – few dips
13. 95% Ethanol for 2 min
14. EA modified for 2 min
15. Absolute alcohol for 2 min
16. Absolute alcohol for 2 min

17. Absolute alcohol for 2 min
18. Xylene for 5 min
19. Xylene till clear
20. Mounted with DPX

Tolbert's criteria given in 1992 were widely used for over three decades in choosing the cell, identifying and counting micronuclei [16].

The parameters for cell inclusion were

1. Intact cytoplasm and relatively flat cells on the slide
2. Little or no overlap with adjacent cells
3. Little or no debris

The criteria for identifying micronuclei were

1. Round smooth perimeter
2. Less than 1/3$^{rd}$ the diameter of the associated nucleus (large and bright nucleus)
3. Staining intensity similar to that of the nucleus
4. Texture similar to that of the nucleus
5. Same focal plane as of nucleus
6. Absence of overlap with, or bridge to, the nucleus

Magnus microscope (MLX-B Plus LED, Magnus Opto Sytems, India) was used to count the micronuclei (Figure 8b). Two experienced oral pathologists counted the micronuclei in 100x and had substantial Inter-observer agreeability of 0.62. The micronuclei from the buccal epithelial cytosmear are shown in 100x (Figure 9).

### 5.3.10 MEASUREMENT OF SALIVARY pH

The unstimulated salivary sample was collected from the study participants in a new sterile container for pH estimation using Eutech pHTestr 20 (Thermo Fisher Scientific, Singapore) (Figure 10). The pH electrode was dipped entirely into the saliva solution, gently stirred and stable pH reading was recorded. The pH tester has a range of -1.00 to 15.00 pH with a resolution of 0.01 pH, two-point calibration and automatic temperature correction. The two-point calibration of the pH tester was done as per the manufacturer's recommendation on weekly basis with two buffers pH 4.01 and 7.00. The pH tester was stored in a storage solution when not in use.

### 5.3.11 QUANTIFICATION OF *STREPTOCOCCUS SALIVARIUS* LEVEL IN UNSTIMULATED SALIVA USING QUANTITATIVE REAL-TIME PCR.

The unstimulated salivary sample was thawed to room temperature. According to the manufacturer protocol, *Streptococcus salivarius* DNA extraction was performed using HELINI Purefast Bacterial DNA Mini spin prep kit (Helini Biomolecules, Chennai, India) (Figure 11a). 1ml of the unstimulated whole saliva sample was centrifuged in a cooling centrifuge (Remi, Vasai, India) at 4°C at 3000 rpm and the 200µl supernatant was used for DNA extraction.

1. 20µl of Protein Kinase was added to this 200µl saliva. Further, 200µl of Binding buffer was also added and mixed well using pulse vortex for 15 seconds. The contents were also mini-centrifuged for a few seconds to bring down drops to the bottom of the tube.
2. 5µl of internal control was added to the above mixture and incubated at 56°C for 15 minutes in a water bath. The elution buffer was also warmed in a water bath.
3. 200µl of absolute ethanol (100%) was added and mixed well by inverting several times.
4. The entire sample was pipetted into the Purefast spin column and centrifuged at 8000rpm for 1 min. The flow-through was discarded and the spin column was placed back into the same collection tube.
5. 500µl wash buffer-1 was added to the Purefast spin column, centrifuged at 10000rpm for 1 minute and the flow through was discarded. The spin column was again placed into the same collection tube.
6. 500µl wash buffer-2 was added to the Purefast spin column, centrifuged at 10000rpm for 1 minute and the flow through was discarded. The spin column was again placed into the same collection tube. This step was repeated twice as per the manufacturer's instructions. The collection tube was discarded.
7. The Purefast spin column was inserted into a fresh 2 ml microcentrifuge tube. Empty spin was done at 12000rpm for 2 min to avoid residual ethanol. The centrifuge tube was discarded.
8. The Purefast spin column was transferred into a fresh 2 ml microcentrifuge tube. 100µl of the pre-warmed elution buffer was added to the centre of the spin column, incubated at room temperature for 2 min and centrifuged at 13000rpm for 1 min. The spin column was discarded and the pure extracted DNA in the microcentrifuge

tube was stored at -20°C. The wash buffers and elution buffer images are shown in Figure 11b.

Helini Streptococcus salivarius Real-time PCR kit (Helini Biomolecules, Chennai, India) was used for quantifying *Streptococcus salivarius*. Preparation of standard curve dilution series:

**Preparation of standard curve dilution series:**

1. 90µl of PCR grade water was pipetted into three 1.5ml microcentrifuge tubes and labelled as QS2 to QS4. (Table 5)
2. 10µl of Positive control-QS1 was Pipetted into tube QS2.
3. It was vortexed thoroughly and spin down briefly.
4. 10µl from tube QS2 was pipetted into tube QS3.
5. It was vortexed thoroughly and spin down briefly.
6. Steps 4 and 5 were repeated to complete the dilution series.
7. 10µl was used per reaction.

**Table 5: Standard curve dilution series**

| Standards | Copies per µl |
|---|---|
| QS-1 | 200000 |
| QS-2 | 20000 |
| QS-3 | 2000 |
| QS-4 | 200 |

For quantitative PCR reactions, the Rotor-Gene Q 2PLEX HRM Real-time PCR system (Qiagen, Germany) was used (Figure 12). A final volume of 25µl was prepared to consist of 10µl of probe PCR master mix, 2.5µl of *Streptococcus salivarius* primer probe mix, 2.5µl of internal control primer probe mix and 10µl of purified DNA. FAM and HEX were the fluorescence dyes used for programming the thermal cycler. For data collection, FAM targeted *Streptococcus salivarius* and HEX targeted the internal control.

The amplification protocol involved Taq enzyme activation at 95°C for 15 min followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at 56°C for 20 sec and extension at 72°C for 20 sec. For qualitative detection, FAM <37 and HEX <31 indicated the detection of *Streptococcus salivarius*.

The Standard DNA of *Streptococcus salivarius* (Helini Biomolecules, Chennai, India) was used to generate the standard curve. Copies of *Streptococcus salivarius* in each sample were calculated based on the above quantification with relevance to the standard.

**Calculation of copies/ml**

The machine indicated copy number was entered into the following formula

$$Result\ (Copies/\mu l) = [Machine\ Result\ (Copies/\mu l) \times Elution\ volume\ (\mu l)] \div Sample\ volume\ (ml)$$

**5.3.12 Statistical Analysis**

Data were subjected to statistical analysis. Kruskal Wallis ANOVA was performed to compare low, medium and high PD groups along with the Mann Whitney U test to compare between the groups using the SPSS version 25.0 (SPSS Inc., Chicago, IL, USA).

## 6. Results:

The mean age of the study participants among the three groups namely low, medium and high PD is given in Table 6. There is no statistically significant difference in mean age among the three study groups. The gender distribution among the three groups is given in Table 7. Both male and female participants are equally distributed in all three study groups. The mean duration of stay around the cell phone towers in years is given in Table 8. Inter-group comparison of weekly usage of EMF emitting devices inside the house is provided in Table 9. There is no statistically significant ($p<0.05$) difference in the mean weekly usage of different EMF-emitting devices inside the house.

**Table 6. The mean age of the study participants among the low, medium and high PD groups**

| Groups | n | Mean ± Std. deviation (years) | p value |
|---|---|---|---|
| Low PD | 20 | 21.80 ± 2.33 | .523 |
| Medium PD | 20 | 22.75 ± 3.70 | |
| High PD | 20 | 21.35 ± 2.68 | |

$p<0.05$ is considered statistically significant

**Table 7. Gender distribution among the low, medium and high PD groups residing around the cell phone towers**

| Groups | Male | Female | Total |
|---|---|---|---|
| Low PD | 10 (50%) | 10 (50%) | 20 |
| Medium PD | 10 (50%) | 10 (50%) | 20 |
| High PD | 10 (50%) | 10 (50%) | 20 |
| Total | 30 | 30 | 60 |

**Table 8. Mean years of stay around the cell phone towers among the low, medium and high PD groups**

| Groups | n | Mean ± Std. deviation (years) | p value |
|---|---|---|---|
| Low PD | 20 | 8.2500 ± 2.8631 | 0.11 |
| Medium PD | 20 | 6.7000 ± 1.6254 | |
| High PD | 20 | 7.4167 ± 1.8381 | |

$p<0.05$ is considered statistically significant

**Table 9. Weekly use of different EMF emitting devices inside the house among the low, medium and high PD groups**

| Groups | n | Mean ± Std. deviation (hours) | p value |
|---|---|---|---|
| Low PD | 44 | 232.6500 ± 137.721 | 0.11 |
| Medium PD | 44 | 285.250 ± 184.380 | |
| High PD | 44 | 312.5500 ± 217.208 | |

$p<0.05$ is considered statistically significant

A statistically significant ($p<0.05$) reduction in unstimulated salivary flow rate is seen in the low, medium and high PD groups (Figure 13). Further, Intra-group comparison also shows a statistically significant ($p<0.05$) reduction in the unstimulated salivary flow rate between the low and high PD groups (Figure 14). There is no statistically significant ($p<0.05$) reduction in salivary pH among, low, medium and high PD groups (Table 10).

**Table 10. Salivary pH comparison among the low, medium and high PD groups**

| Groups | n | Mean ± Std. deviation (pH) | p value |
|---|---|---|---|
| Low PD | 20 | 6.76 ± 0.22 | 0.780 |
| Medium PD | 20 | 6.73 ± 0.34 | |
| High PD | 20 | 6.79 ± 0.32 | |

$p<0.05$ is considered statistically significant

The inter-group comparison between the low, medium and high PD groups shows a statistically significant ($p<0.05$) increase in the buccal epithelial micronuclei count. (Figure 15). The intra-group comparison shows a statistically significant ($p<0.05$) increase in buccal epithelial micronuclei count between the low and medium PD groups (Figure 16).

Regarding the Real-Time PCR for *Streptococcus salivarius*, the Coefficient of Determination (R) = 0.9852 is indicating the standard value is reliable (Figure 17).

The graphical curve between the Threshold cycle (Ct) and fluorescence in Real-Time PCR for standard indicates PCR has happened correctly without contamination (Figure 18). The amplification happened around 25 cycles in low PD (Figure 19), medium PD (Figure 20) and high PD groups (Figure 21).

Based on the Ct value and the calculated values, there is a statistically significant ($p<0.05$) decrease in *Streptococcus salivarius* levels in the medium and high PD groups compared to the low PD group (Figure 22). Intra-group comparison between the low and high PD groups reveals a statistically significant ($p<0.05$) decrease in *Streptococcus salivarius* level in the high PD group (Figure 23).

## 7. Discussion:

The continuous exposure to electromagnetic field (EMF) radiation from cell phone towers may possibly have an influence on public health among the population residing near and around them [17]. The power density-based classification is preferred for the following discussed reasons. As per the FCC's RF exposure guidelines, the maximum permissible exposure level to the general public is based on PD, which is 580 $\mu W/cm^2$. This PD is several times greater than the RF value found around the cell phone towers. The FCC-adopted guidelines are identical to those of

the National Council on Radiation Protection and IEEE [18]. Hence, we prefer power density over EF and MF. The following equation relates PD, EF and MF:

$$PD = E \times H$$

where *PD* is power density, *E* is electric field strength, and *H* is magnetic field strength. The unit of PD for the above equation is W/m$^2$. As 1 mW/cm$^2$ has the same power density as 10 W/m$^2$, the following equation can be used to obtain these units directly.

$$PD = 0.1 \times E \times H$$

where *PD* is power density, *E* is electric field strength, *H* is magnetic field strength and 0.1 is the conversion from 10 W/m$^2$ to 1 mW/cm$^2$. The unit of PD for the above equation is mW/cm$^2$ [19]. In the present study, we prefer μW/cm$^2$ similar to FCC exposure guidelines.

There are two closely placed terms, namely radiation intensity and power density. Power density is the rate of flow of electromagnetic energy or power per unit area. It is expressed as μW/cm$^2$. The radiation intensity is defined as the power per unit solid angle. It is expressed in W/sr [10]. In the present study, we measured the power density and not the radiation intensity.

Another relevant parameter, the input power of the cell phone tower can be important in terms of EF, MF and PD. However, the PD-based classification is practical and easier to apply than a classification based on the power input to the cell phone tower. The attenuation by brick walls and other materials will change the PD regardless of the input power provided to the base station. Hence, PD measurements inside the house are more practical and relevant for conducting epidemiological studies. Hence, PD-based classification is a preferred method rather than input power-based classification.

According to the FCC guidelines for cellular antenna sites, the majority of cell phone towers operate at an Effective Radiated Power (*ERP*) of 100 W or less, which corresponds to a Total Radiated Power (TRP) of 5–10 W [18]. The *ERP* of an antenna is defined as the product of power transmitted to the antenna and antenna gain. It is measured in W. ERP is calculated by the following Equation:

$$ERP = Pt \times Ag$$

where *ERP* is the Effective Radiated Power, *Pt* is the total power transmitted by the antenna and *Ag* is the antenna gain; $Pt = Radiofrequency\ power - cable\ loss$ [20 ].

The TRP is usually lower than the ERP due to conductor loss, dielectric loss and unwanted surface waves in the cell phone tower antenna material [21]. In the present study, ERP and TRP were not measured. Rather, PD was measured inside the houses around the cell phone towers and used in developing a classification.

Saliva plays a major role in maintaining homeostasis in the oral cavity. The functions of saliva include lubrication, bolus formation, soft and hard tissue protection against bacterial, viral and fungal infections, dental remineralization and buffering action. The quantitative alteration could lead to local changes like caries, candidiasis, mucositis, etc. The qualitative alteration could lead to dysphagia, halitosis, weight loss, etc. Unstimulated whole saliva is an accurate method to analyze the salivary gland status (22).

In a study conducted by Singh K et al (2016), a statistically significant reduction in the stimulated salivary volume and buffering capacity is observed among the 40 study participants residing in the proximity of around 4 randomly selected cell phone towers at Rajasthan, India. The distance-based grouping was adopted with one group residing in proximity to the cell phone tower and the other group residing 1km away from the cell phone towers. Further, pH was measured using pH strips [8]. These results of the study cannot be compared with the present study as few major differences exist. The present study used PD-based classification unlike distance and the PD was measured inside the house taking into consideration of attenuation by building materials. In addition, all the EMF radiation producing devices inside the house and their weekly hours of use were also considered using a questionnaire and included in the present study. In the present study, a statistically significant reduction in unstimulated salivary flow rate is observed in the high PD group. This reflects there could be reduction in the protective functions of saliva with respect to salivary flow rate among the population residing around the cell phone towers with high PD. This could be due to electro hypersensitivity with downregulation of the parasympathetic nervous system and upregulation of the sympathetic nervous system [23]. This area needs further research for a better understanding of the effects of EMF radiation on the parasympathetic and the sympathetic nervous systems.

In the study conducted by Singh K, colorimetric pH strips were used for measuring pH and there is a reduction in buffering capacity among the population residing close to the cell phone towers [8]. However, pH strips require normal colour vision to be used in research. Further, pH strips also show variable degrees of error according to the study done by Methany et al [24]. In the present study, a pH tester with double decimal was used instead of pH strips and the device was calibrated as per the manufacturer's recommendation. Hence, the results are reliable in the present study. The mean pH of the study participants among the groups is slightly acidic, 6.7. However, this pH is higher than the critical pH of 5.5 and hence cannot induce caries. The pH of different commercially available toothpaste in India is studied by Pragya Soni [25]. According to this present study, 67.5% used fluoridated toothpaste. Hence, despite these differences, there is no statistically significant difference in the salivary pH among the low, medium and high PD groups.

Micronuclei are small membrane-bound DNA structures encapsulated by an envelope and separated from the nucleus [26]. Micronuclei assay in humans is used for investigating the genotoxic effects of environmental factors [27]. Thamilselvan S conducted a study on buccal epithelial micronuclei among the population residing around three cell phone towers within a radius of 10-25 m. The results of the study show 49.54% of the micronuclei is seen in the younger age group between 0-20 years [9]. In the present study, a statistically significant ($p<0.05$) increase in micronuclei is seen among the lower and medium PD groups. This study is unique as it considered not only the power density inside the house, but also all the EMF emitting devices inside the house among healthy adults aged between 18-30 years and hence reliable. Despite the increase in micronuclei count between the groups, it is under 1% and it is not consistent with the study done by Thamilsevan S et al. [9]. Micronuclei correlated with age and women have higher frequencies than men [28]. Micronuclei and the diet have two different schools of thought. Kotova et al. (2014) proposed that nonvegetarians have a higher frequency of micronuclei as compared to vegetarians, which indicates a higher level of DNA damage in non-vegetarians. Fenech M observed that for the age group between 61-90 years, micronuclei frequency is the same regardless of the vegetarian or non-vegetarian diet. Micronuclei frequency is significantly lower in vegetarians aged between 41-60 years compared to non-vegetarians. Most importantly, MN frequency is significantly higher in vegetarians between the age of 20-40 years compared to non-vegetarians [27]. In the present

study, 85% of the study participants are non-vegetarians and the age range is 18-30 years. Hence, micronuclei are expected to be lower in all study participants. There is a statistically significant ($p<0.05$) increase in micronuclei in medium PD group compared to the low PD group. This implies there is a minimal contributory role of EMF radiation from cell phone towers in inducing micronuclei formation. This area needs further studies to understand the role of EMF radiation in inducing micronuclei formation.

*Streptococcus salivarius* is of particular importance to humans. They are the first bacteria to colonize the oral mucosa in the first few days after birth and are maintained as the dominant population in the human oral cavity throughout life. They are commensals and occasionally cause opportunistic infections in humans. The organism impacts human health through the effects on the stability of microbiota composition, bacterial interference and host interaction. In the present study, a statistically significant ($p<0.05$) reduction in *Streptococcus salivarius* is observed in the high PD group compared to the low PD group. This reduction needs consideration as *Streptococcus salivarius* has important protective functions namely preventing initial adhesion of cariogenic *Streptococcus mutans* to tooth sites and inhibiting epithelial colonization of periodontal pathogen *Aggregatibacter actinomycetemcomitans* [29]. These protective functions may be lost due to chronic exposure to high PD from the cell phone towers due to a reduction in *Streptococcus salivarius* level. Ksenia B et al (2022) observed the protective role of *Streptococcus salivarius k12*, an oral probiotic, exhibiting a significant reduction in plaque accumulation at 4 and 6 weeks in the probiotic group in a randomized clinical trial [30].

The weekly usage of other EMF emitting devices inside the house was also considered in the present study (hours) and there is no statistically significant difference among the low, medium and high PD groups. It is similar in all three groups.

Further, the study participants were young healthy males and females in the age group 18 - 30 years without systemic diseases, without medication, no alcohol, areca nut or tobacco habits, residing 6 - 8 years around the cell phone towers in all three groups. The only difference is the PD between the groups. This indicates that the results of the present study could be a true outcome of the effects of the PD from the

cell phone towers on unstimulated whole salivary flow rate, buccal epithelial micronuclei and *Streptococcus salivarius* level.

## 8. Summary and conclusions

The present study estimated and compared the unstimulated salivary flow rate, pH, *Streptococcus salivarius* level and buccal epithelial micronuclei changes among the human population residing around the cell phone towers using PD-based classification. There is a statistically significant ($p<0.05$) decrease in unstimulated salivary flow rate in high PD group, measured by the gravimetric method. A statistically significant ($p<0.05$) reduction in *Streptococcus salivarius* level is seen in the high PD group. A statistically significant ($p<0.05$) increase in micronuclei count is observed in buccal epithelial cytosmear in the medium PD group.

To conclude, there is no standardized method for conducting research among the population residing around cell phone towers. Arbitrary distance-based classification is preferred for its convenience of use. However, several other cell phone tower characteristics such as the number of antennas, type of antennas, roof-mounted or ground-based antenna, transmitted power, input power, electric field strength, magnetic field strength, and power density must also be considered. In addition, attenuation by the building materials, EMF radiation from different sources inside the house, hours of stay inside the house and duration of stay around the cell phone towers must be considered to find the effects of EMF radiation from cell phone towers.

This study is unique as it considered the number of antennas, type of antennas, roof-mounted or ground-based cell phone towers, electric filed strength, magnetic field strength, and power density around the cell phone towers in developing the PD-based classification. Further, PD is measured inside the house of study participants taking into consideration of attenuation by building materials, different EMF emitting devices and weekly hours of use inside the house, hours of stay inside the house and duration of stay around the cell phone towers. Hence, the changes seen in the present study concerning the unstimulated salivary flow rate, Streptococcus salivarius level and buccal epithelial micronuclei count are true effects of EMF radiation from cell phone towers. Further, more standardized methods are needed to study the effects of EMF radiation from cell phone towers on the human population.

## 9. Figures

Figure 1. Geographical mapping of cell phone towers using Garmin etrex 20x device. The short white arrow indicates the cell phone tower

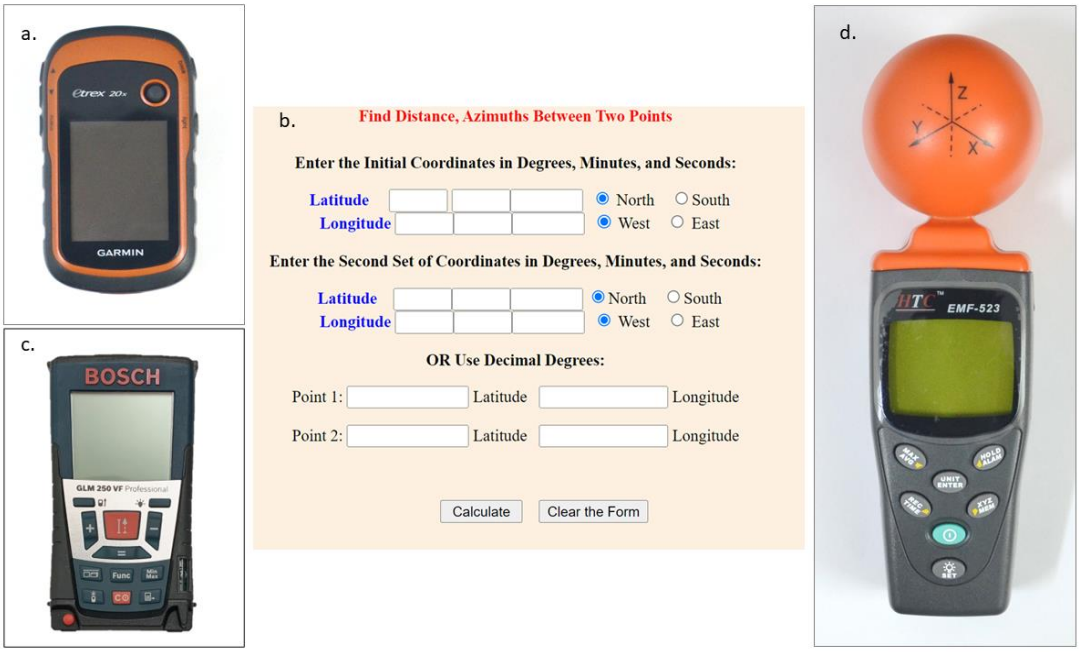



Figure 2. **a.** Garmin etrex 20x device to identify the cell phone tower coordinates; **b.** FCC Online conversion tool for calculating distance between two cell phone towers based on two coordinates; **c**. Bosch GLM 250 VF Professional range finder to measure the distance around the cell phone tower; **d** HTC EMF-523 to measure EF, MF and PD.

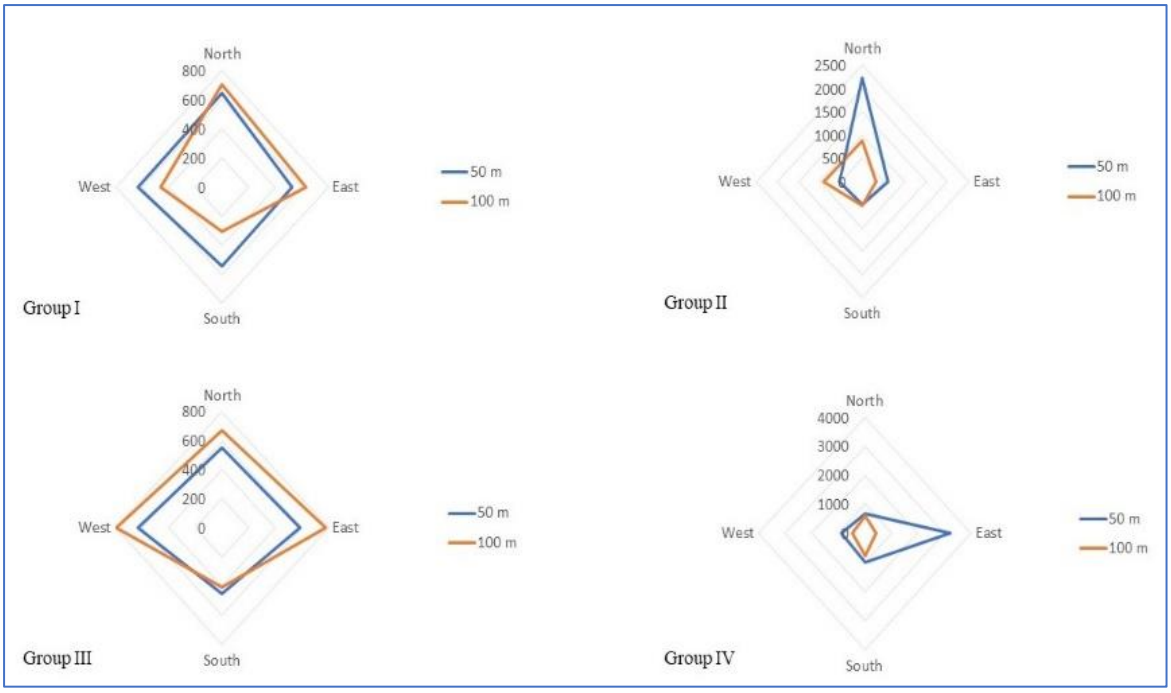



Figure 3. Radar chart showing electric field strength distribution around cell phone towers among different study groups.

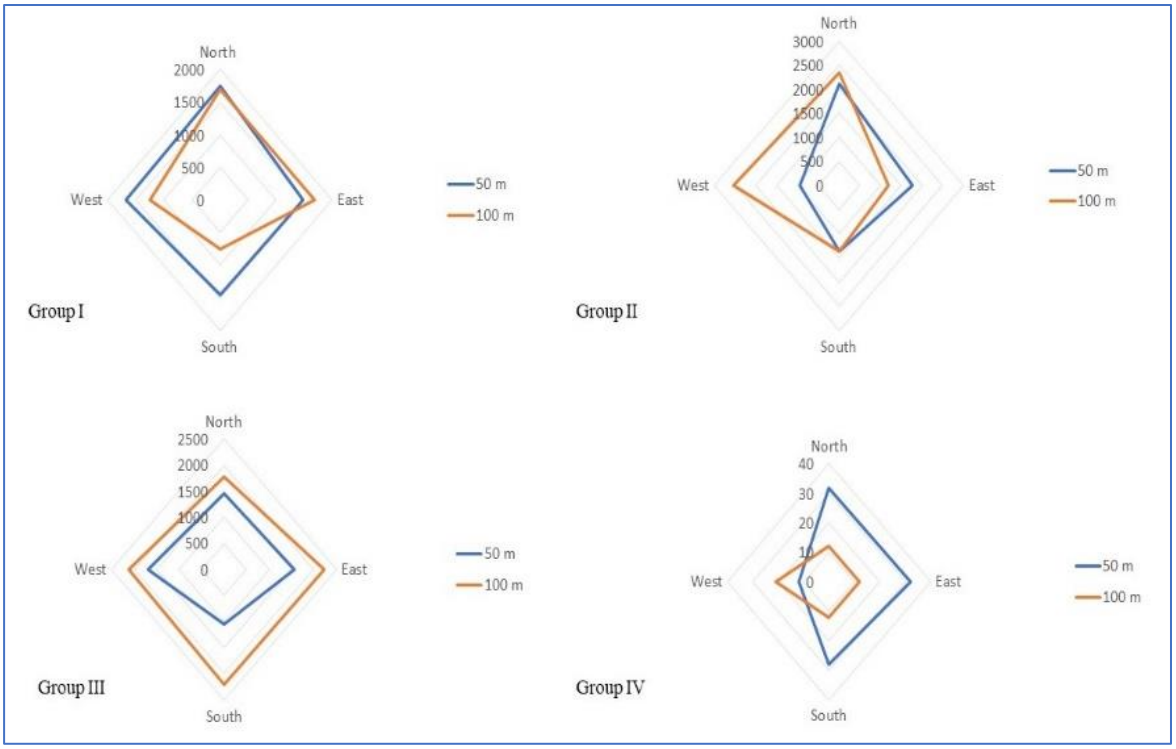



Figure 4. Radar chart showing magnetic field strength distribution around cell phone towers among different study groups.

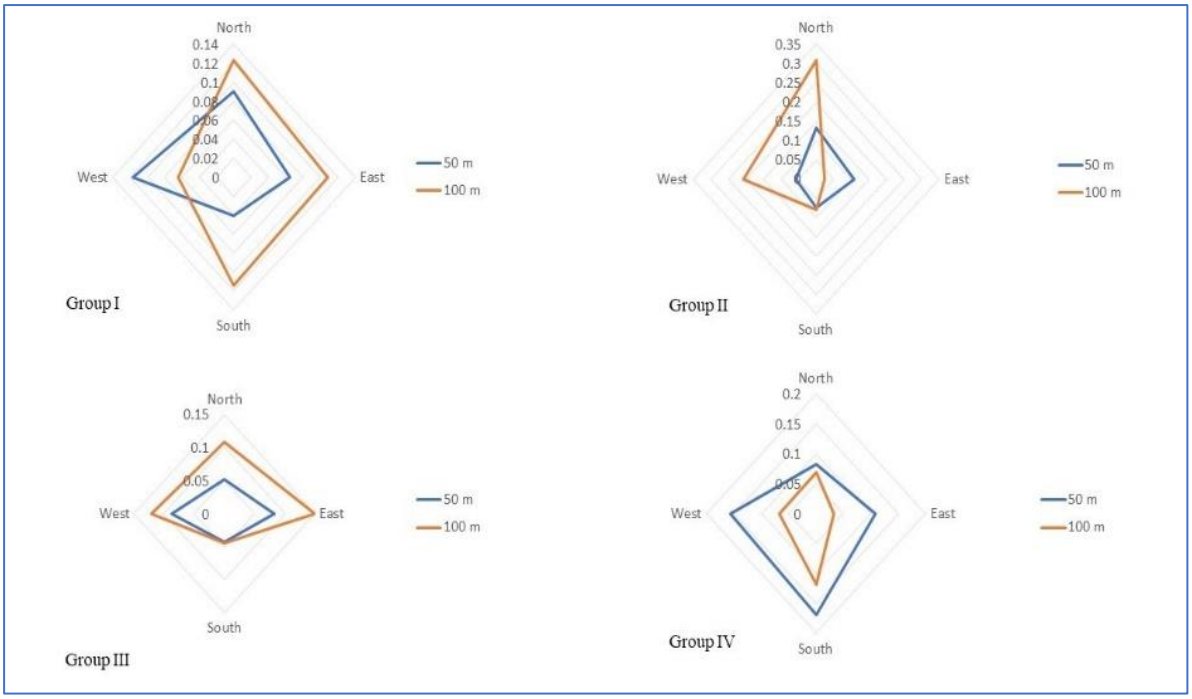



Figure 5. Radar chart showing power density distribution around cell phone towers among different study groups.

Figure 6. a. The pre weight of the container; b. The post weight of the container. Both the measurements were measured using precision balance (Wensar PGB 220, India)

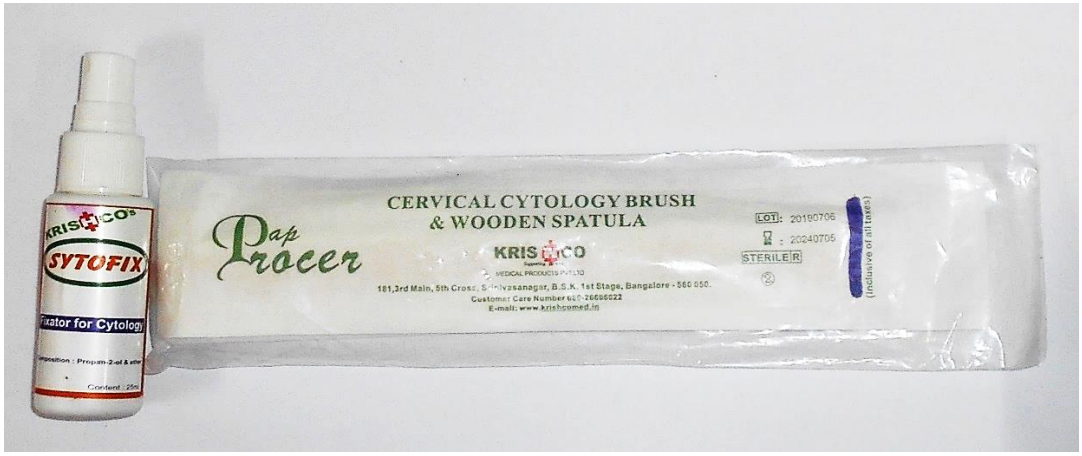



Figure 7. The buccal epithelial cytosmear was obtained from the left buccal mucosa using the cytobrush (Pap Procer, Bangalore, India) and fixed with styofix.

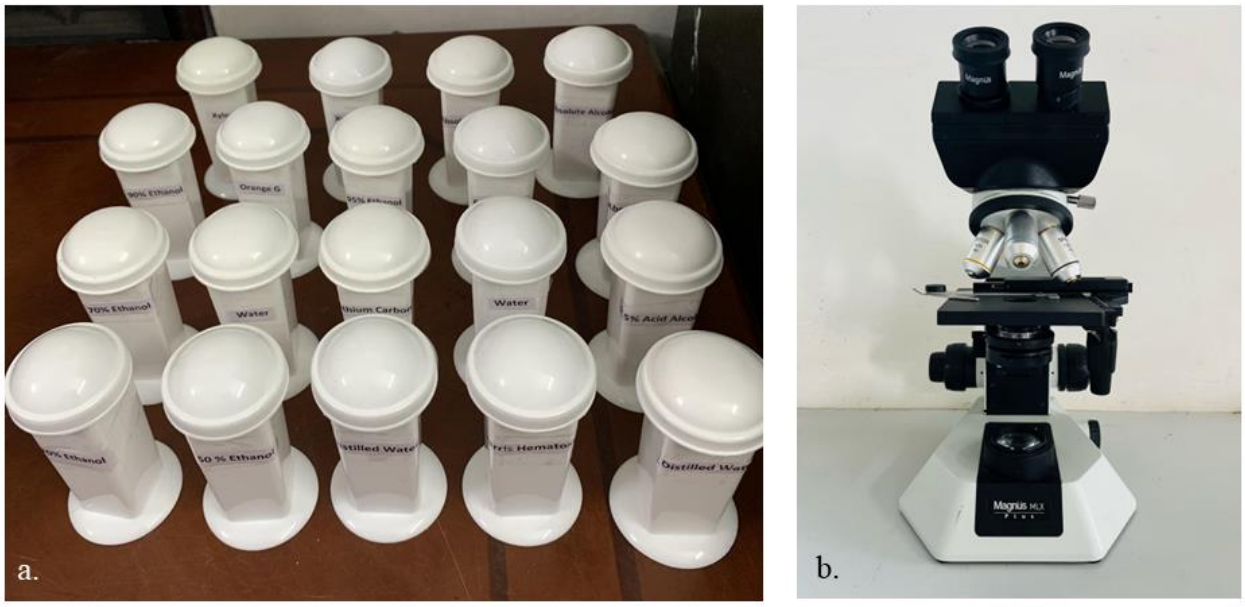



Figure 8. a. PAP staining to visualize micronuclei; b. Magnus microscope (MLX-B Plus LED, Magnus Opto Sytems, India) was used to count the micronuclei;

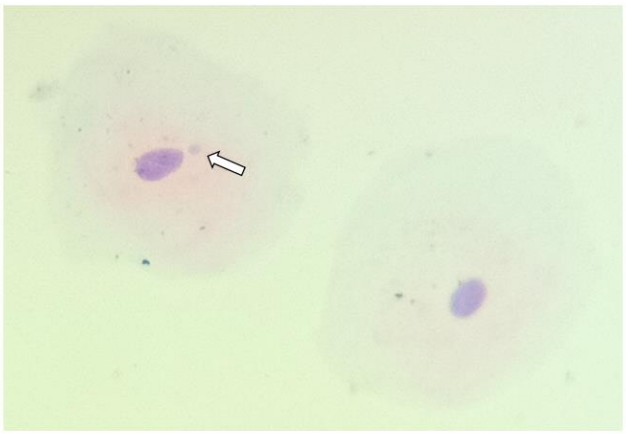


Figure 9. The short white arrow indicates micronuclei from the buccal epithelial cytosmear in 100x

Figure 10. Eutech pHTestr 20 used for salivary pH estimation

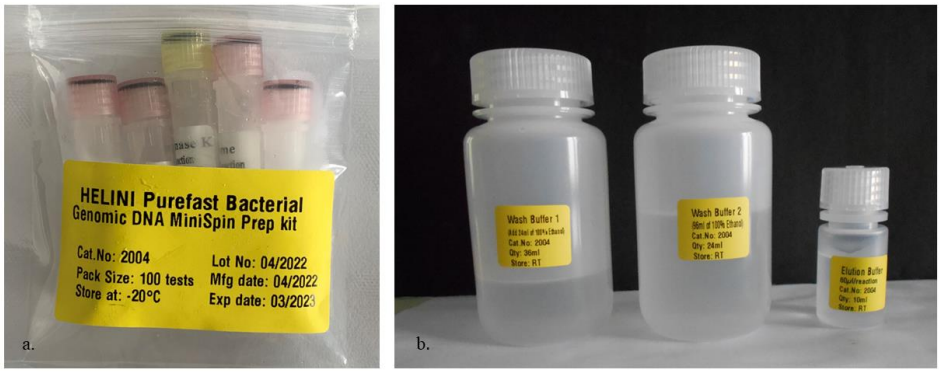
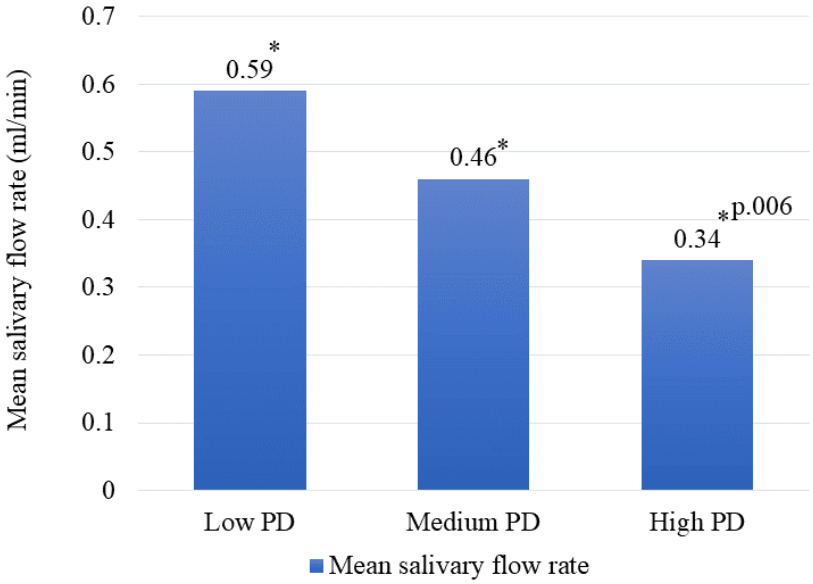


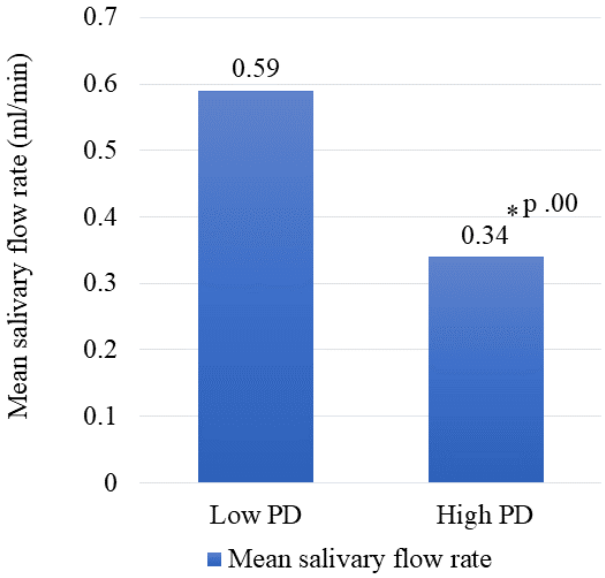

Figure 11. a. Helini Purefast Bacterial Genomic DNA MiniSpin Prep Kit; b. Wash buffer 1 and 2 along with Elution buffer

Figure 12. Rotor-Gene Q 2PLEX HRM Real time PCR system (Qiagen, Germany) was used for *Streptococcus salivarius* quantification



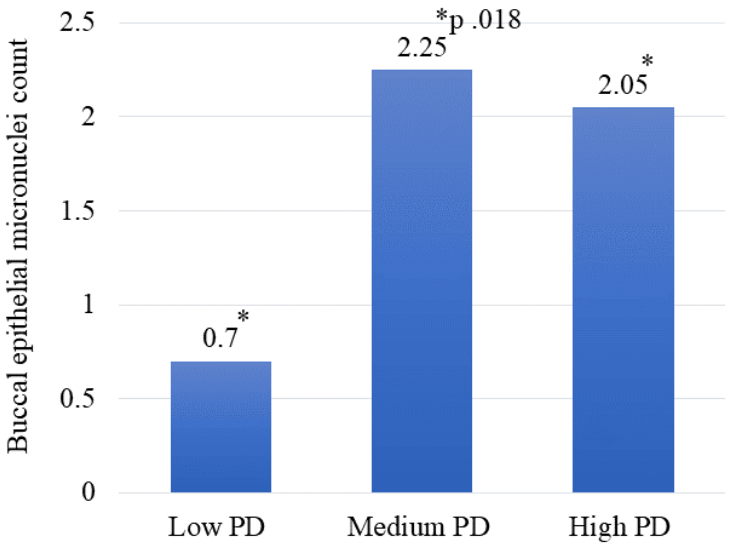

* There is statistically significant p<0.05) reduction in salivary flow rate among the high and medium PD group compared to the low PD group

Figure 13. Inter-group comparison of unstimulated salivary flow rate among the 3 groups



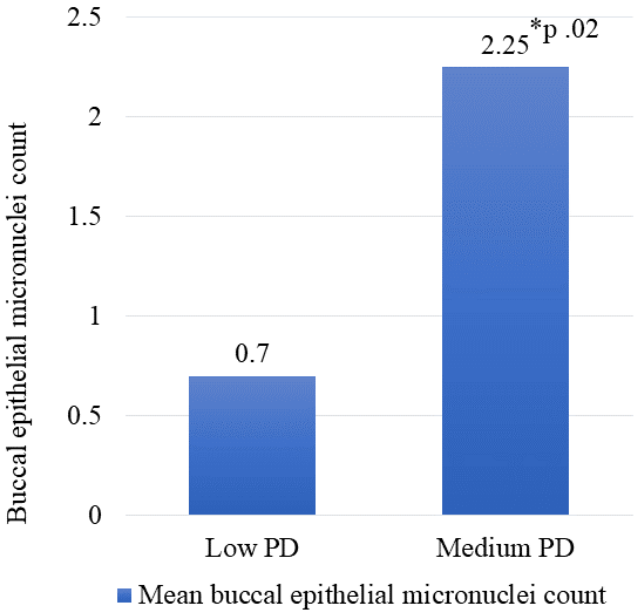

* There is statistically significant p<0.05) reduction in salivary flow rate in high PD group compared to the low PD group

Figure 14. Intra-group comparison of unstimulated salivary flow rate between low and high PD groups




* There is statistically significant p<0.05) increase in buccal epithelial micronuclei count in the medium and high PD group compared to the low PD group

Figure 15. Inter-group comparison of buccal epithelial micronuclei count among the low, medium and high PD groups




* There is statistically significant p<0.05) increase in mean buccal epithelial micronuclei count in medium PD group compared to the low PD group

Figure 16. Intra-group comparison of buccal epithelial micronuclei count between the low and medium PD groups

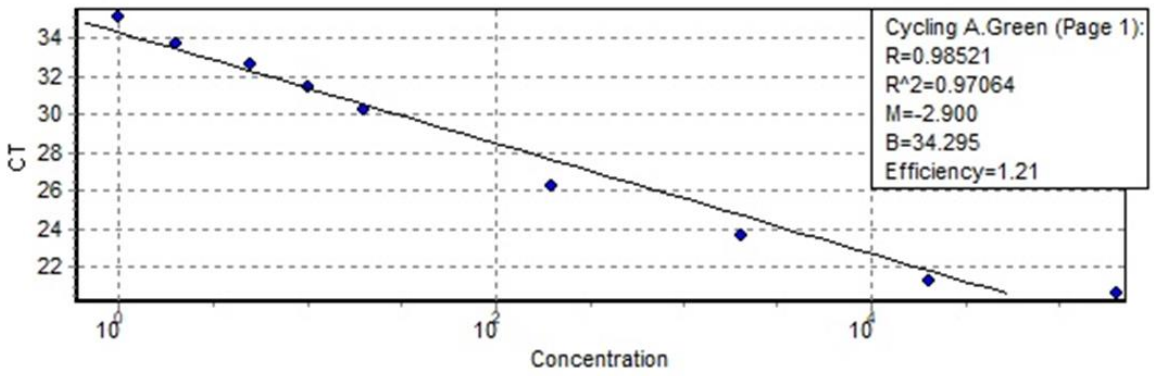



Figure 17. The Co-efficient of Determination (R) = 0.9852 is indicating the standard value is reliable

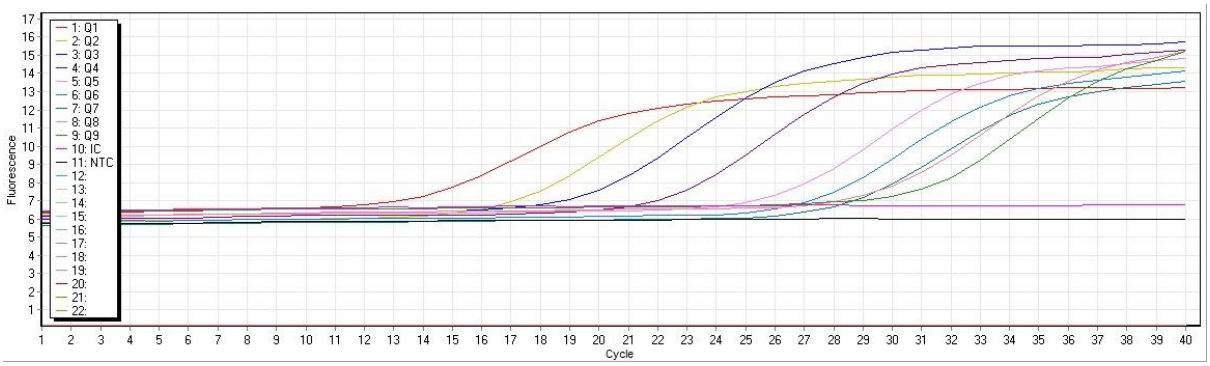



Figure 18. Real time PCR standard curve showing threshold cycles ($T_C$) and florescence. No template control (NTC) indicate Real time PCR is conducted properly without any contamination

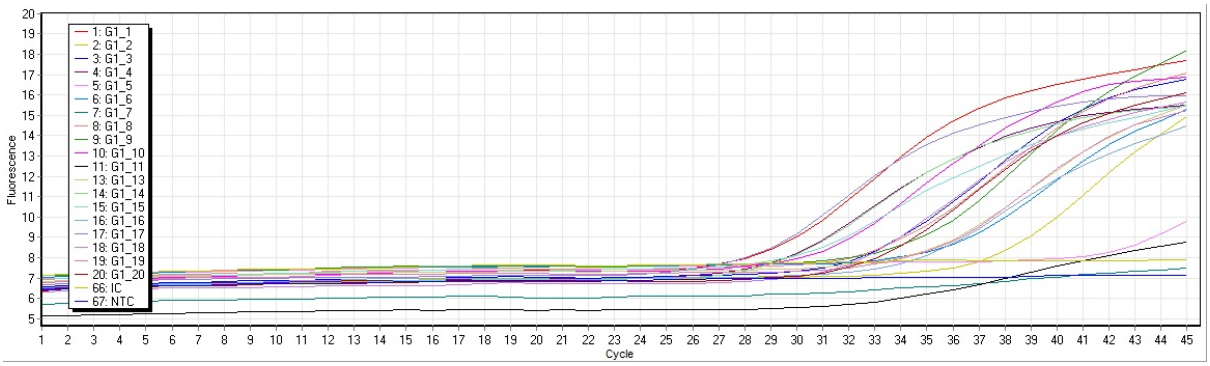



Figure 19. Real time PCR showing threshold cycles ($T_C$) and florescence in low PD group

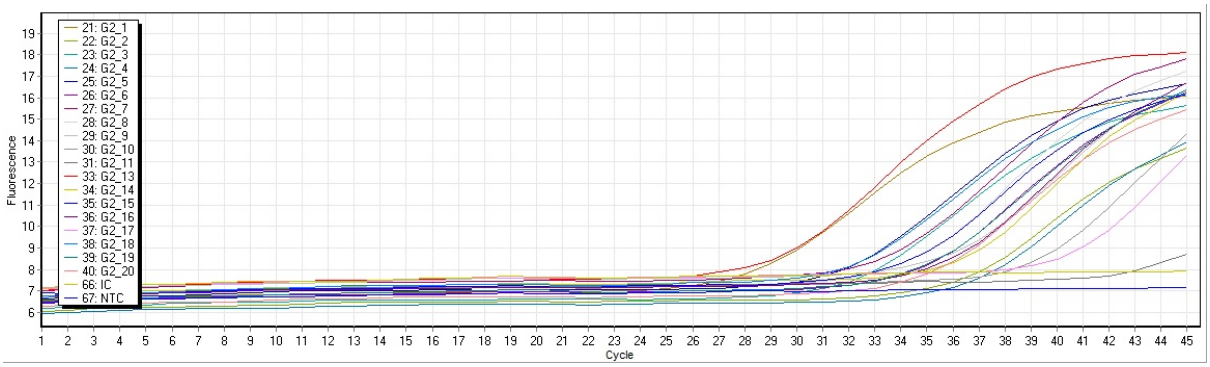



Figure 20. Real time PCR showing threshold cycles ($T_C$) and florescence in medium PD group

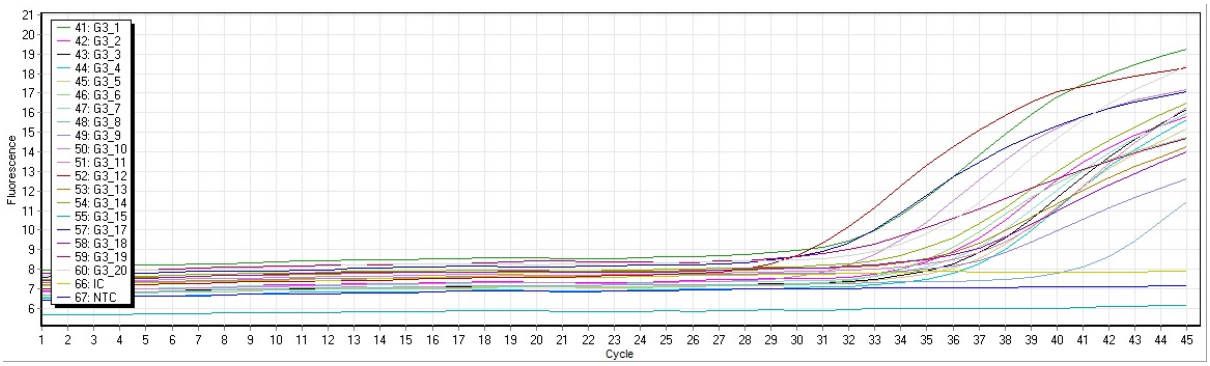
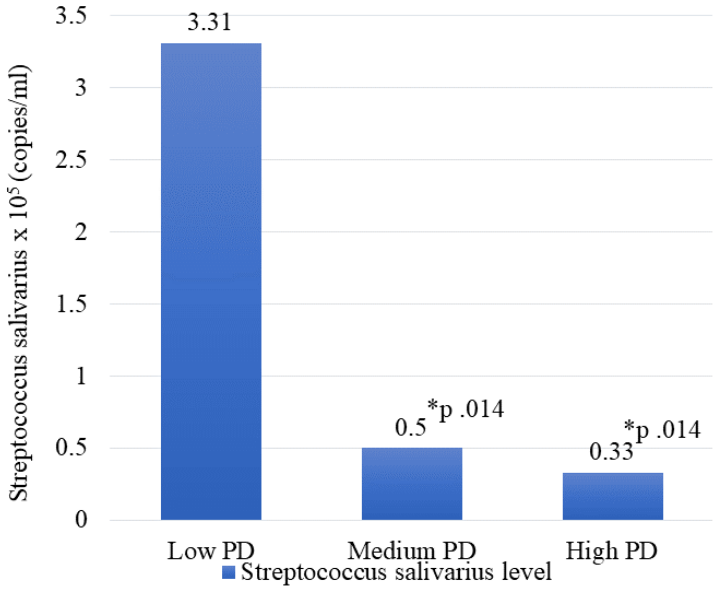
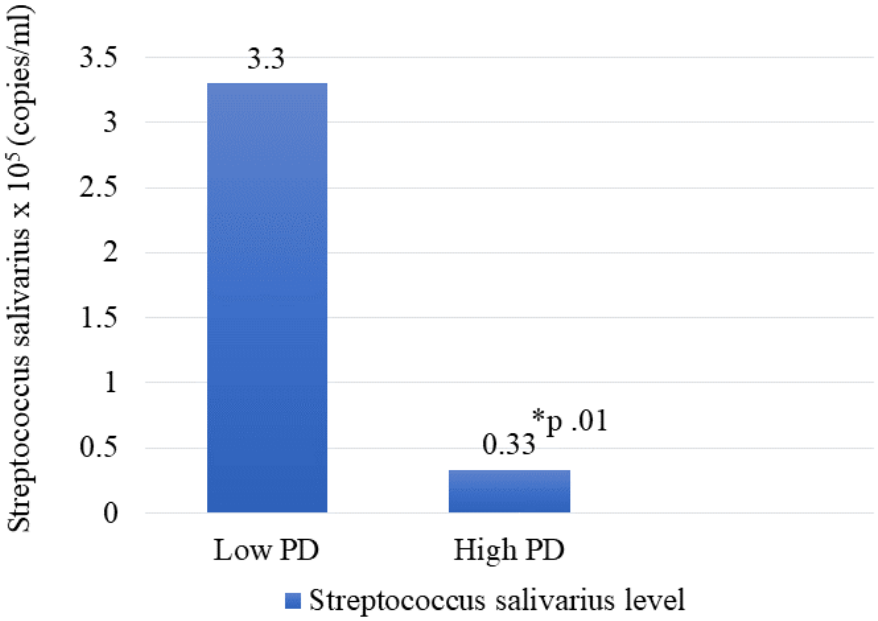



Figure 21. Real time PCR showing threshold cycles ($T_C$) and florescence in high PD group




* There is statistically significant p<0.05) decrease in Streptococcus salivarius level in high and medium PD group compared to the low PD group

Figure 22. Inter-group comparison of Streptococcus salivarius level among the low, medium and high PD groups




* There is statistically significant p<0.05) decrease in Streptococcus salivarius level in high PD group compared to low PD group

Figure 23. Intra-group comparison of Streptococcus salivarius level between the low and high PD groups

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